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# LSD1- induced signaling mechanisms inhibition sensitizes oral cancer for chemotherapy and immunotherapy

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BOSTON UNIVERSITY

HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

THESIS

**LSD1- INDUCED SIGNALING MECHANISMS INHIBITION SENSITIZES  
ORAL CANCER FOR CHEMOTHERAPY AND IMMUNOTHERAPY**

by

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Submitted in partial fulfillment of the requirements for the degree of

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In the Department of Endodontics

2025

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## **DEDICATION**

I would like to dedicate this work to my family.

## **ACKNOWLEDGMENTS**

I would like to thank for family for their support throughout this journey and for pushing me towards newer limits. Thank-you to Dr. Manish Bais , Dr. Guoxian Wei and Dr. Sami Chogle for their research mentorship and guidance. Thank-you to all the current and past lab members for their help and support. I would like to thank my attendings from Harlem Hospital for encouraging me towards the wonderful specialty in endodontics. Special thank you to Dr. Joanne Saint- Paul, Dr. Michael Bolden, Dr. Circe Lassegue, Dr. Zahra Omar and Dr. Albert Granger who were pivotal in my professional and personal growth. A special thank-you to Jay Yasin for his encouragement and support.

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**KISA IQBAL**

**Boston University**, Henry M. Goldman School of Dental Medicine, 2025

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**ABSTRACT**

**Introduction:** Lysine-specific demethylase 1 (LSD1) is a nuclear histone demethylase. Our work shows that LSD1 expression progressively increases with tumor grade and stage in clinical oral squamous cell carcinoma (OSCC). The goal is to evaluate the LSD1 mechanism in OSCC by proteomics analysis and its application for immunotherapy and chemotherapy.

**Methods:** The animal experiments were approved by the Institutional Animal Care and Use Committee, Boston University. The conditional LSD1 knockout mice (K14Cre-*Lsd1*; *Lsd1*<sup>-/-</sup>) and littermates (*Lsd1*<sup>WT/WT</sup>) were treated with 4-Nitroquinoline 1-oxide (4NQO) followed by histology and proteomics analysis. Next, 4NQO-treated mice for 20 weeks were subjected to LSD1 inhibitor (SP2509) alone or in combination with Hippo signaling regulator YAP inhibitor (Verteporfin), anti-PD-1 or anti-PD-L1 antibodies for five weeks, followed by IHC and mRNA expression analysis.

**Results:** Global unbiased proteomics analysis of 4NQO-induced OSCC from LSD1<sup>-/-</sup> compared to LSD1<sup>WT/WT</sup> (n=7/condition) showed inhibition of 40 differentially regulated proteins, reduction of canonical pathways signaling including EIF2, mTOR, VEGF, Integrin, ERK/MAPK, Glucocorticoid Receptor Signaling, Acute Phase Response and oxidative phosphorylation evaluated by IPA analysis. LSD1 inhibition attenuates EGF-, YAP-, and pro-inflammatory cytokine-induced pathways involved in oral cancer cell proliferation, migration, and oncogenic transformation. These pathways are validated in HSC-3 and CAL27, and LSD1 knockout OSCC mice. Next, studies in OSCC mice showed that the topical application of LSD1 inhibitor in combination with YAP inhibitor provides sensitivity to OSCC as compared to a single drug alone. LSD1 and YAP co-regulate each other. Finally, genetic, or pharmacological LSD1 inhibition showed to promote PD-L1 expression, thereby sensitizing to the combination of LSD1 inhibitor to anti-PD-1 therapy. Thus, LSD1 inhibition sensitizes to chemotherapy and immunotherapy combinations.

**Conclusion:** We showed for the first time that blocking LSD1 inhibits OSCC, a feed-forward loop oncogenic protein that exists in OSCC and promotes anti-tumor immunity. Next, the detailed mechanism of LSD1 in vitro and in vivo is being evaluated for application in chemotherapy and immunotherapy.

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## LIST OF ABBREVIATIONS

BME.....	Beta- Mercaptoethanol
CoREST .....	Corepressor Element Silencing Factor
DMEM.....	Dulbecco's Modified Eagle's Medium
EGF.....	Epidermal Growth Factor
EMT .....	Epithelial-mesenchymal transition
HI- FBS.....	Heat Inactivated Fetal Bovine Serum
HPV.....	Human Papillomavirus Virus
HSC-3 .....	Human Squamous Cell Carcinoma
LSD1.....	Lysine Specific Demethylase 1
4-NQO.....	4-Nitroquinolone-1-Oxide
OSSC.....	Oral Squamous Cell Carcinoma
PBS .....	Phosphate Buffered Saline
qRT- PCR.....	Quantitative Real Time Polymerase Chain Reaction
SCC.....	Squamous Cell Carcinoma

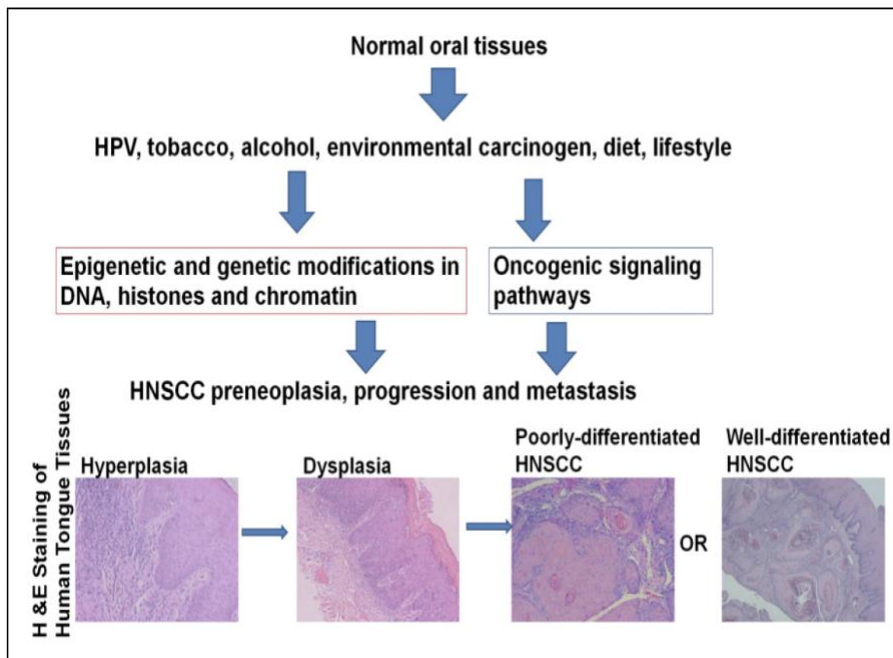


## INTRODUCTION

### 1.1 Oral Squamous Cell Carcinoma

The most recent statistic reports 54,000 new cases and 11,230 deaths related to oral cavity and pharynx cancers (1). Incidence of cancer of the oral cavity and pharynx have continued to increase by 1% per year (1). Oral squamous cell carcinoma (OSSC) is the most common head and neck cancer. OSSC treatment of oral cancer may require chemotherapy, radiotherapy surgery or a combination of these (4). Many patients are resistant to these treatments which may account for the bleak 5- year survival rate of only about 65% (3). Modifications of the genome through means of epigenetic modifications can cause resistance to certain treatments in patients diagnosed with OSCC. The mechanisms of these epigenetic modifications are not well established.

Oncogenic factors including human papillomavirus (HPV), alcohol, tobacco, environmental carcinogens, diet, and lifestyle changes can lead to epigenetic and genetic modifications in DNA, histones, and chromatin (Figure 1). Furthermore, these factors can facilitate the activation of oncogenic signaling pathways.

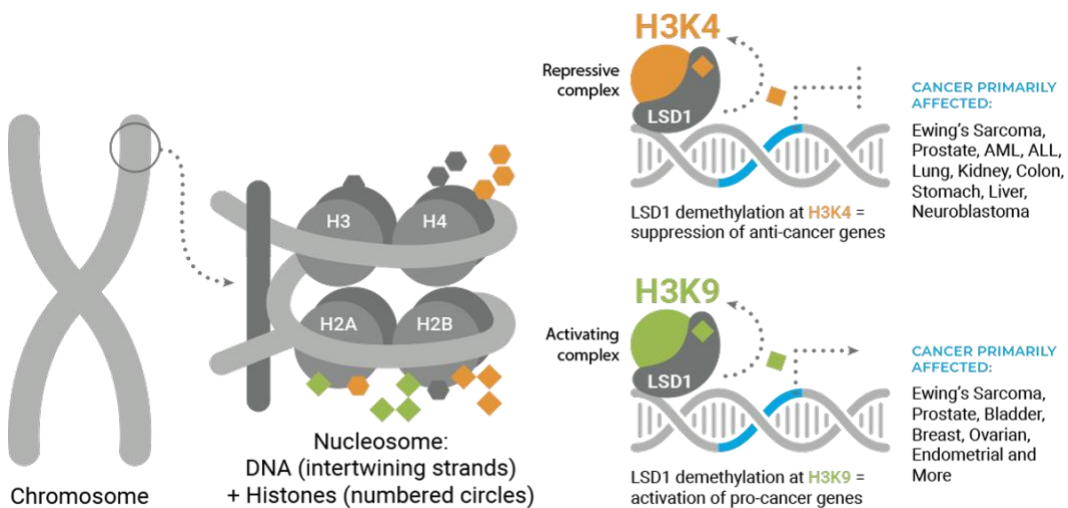


**Figure 1.** Schematic highlighting the potential effects of HPV, tobacco, alcohol, environmental carcinogens, diet, and lifestyle-related changes on normal tissues leading to epigenetic and genetic modifications. Figure taken from our labs previously published work (9).

In the bottom panel of Figure 1, hematoxylin and eosin staining of human tongue tissues display the histologic changes to these tissues. Epigenetic and genetic modifications of DNA, histones, chromatin and oncogenic signaling pathways can all contribute to the progression of head and neck squamous cell carcinoma (HNSCC) from a state of hyperplasia, dysplasia to poor and/or well-differentiated HNSCC.

## 1.2 Lysine- Specific Demethylase -1 (LSD1) and Epigenetics

Lysine-specific demethylase 1 (LSD1), encoded by the *KDM1A* gene, is an epigenetic regulator. This protein is part of the flavin adenine dinucleotide (FAD)- dependent amine oxidase family and has two main functions (17). One of its key roles is the demethylation of the histone H3 tail (5). Its other role involves acting as a scaffolding protein in epigenetic complexes. LSD1 is involved in global hypomethylation in the cancer genome (5).



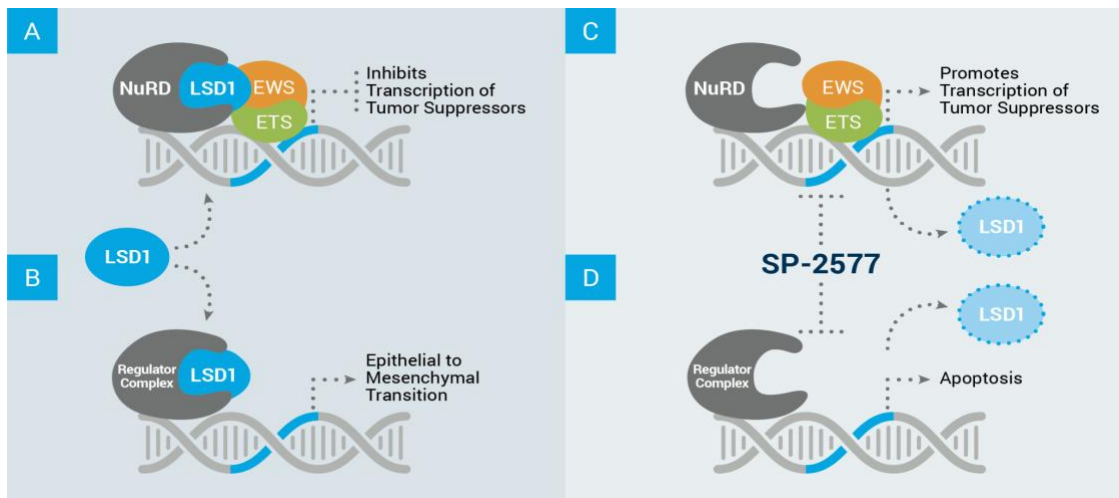
**Figure 2.** Schematic displaying LSD1's effect on DNA demethylation at H3K4 and H3K9 sites. Figure adopted from (5).

LSD1 is a demethylase protein, meaning it can remove methyl groups (-CH<sub>3</sub>) also known as methyl marks from nucleosomes. Nucleosomes are structures involving the

intertwining of 147-base pair stretches wrapped around a histone octamer (9). This octamer involves a pair of H2A, H2B, H3 and H4 histones. Lysine amino acids found on histone structures are a key site in chemical modifications. LSD1 has the capacity to remove or “erase” methyl marks at lysine (denoted by K) as sites 4 and/or 9. LSD1 can act on lysine site 4 on histone 3 (H3K4) or lysine site 9 on histone 3(H3K9). Depending on where the LSD1 acts, chromatin can be in a “heterochromatin” state characterized by inactive genes, tight condensation of genetic material and late replication. Alternatively, chromatin may also be in a “euchromatin” state highlighting its open state and active transcription of certain genes.

At the level of the nucleosome, LSD1 can demethylate at H3K4 or H3K9 on histone 3 which can play a role in different forms of cancer (see Figure 2). If site H3K4 is demethylated, LSD1 forms a repressive complex with other proteins resulting in the suppression of anti-cancer genes. These can result in cancers such as AML, ALL, lung, kidney, colon, and many others. By contrast, if LSD1 demethylates at the H3K9 site, it forms an activating complex with other proteins resulting in the activation of pro-cancer genes. The cancers involved include breast, prostate, bladder, ovarian and others. This highlights LSD1's unique role; it has a dual and context dependent role in the transcription or lack thereof of gene transcription (6). Of note, each lysine amino acid can have three methyl marks. LSD1 can effectively remove mono or di-methylated groups

without affecting trimethylation (10). The number of methyl groups removed can also have a key role in gene expression.



**Figure 3.** LSD1 scaffolding properties driving cancer growth in Ewing Sarcoma and the effect of SP-2577 on these complexes. Figure adopted from (5).

In the context of Ewing Sarcoma, LSD1 can interact with other proteins to form the nucleosome remodeling deacetylase complex (NuRD) (5). When LSD1 is a part of this NuRD complex, there is an inhibition of transcription of tumor suppressor genes (Figure 3, panel A) (5). LSD1 can also interact with a regulator complex to transform healthy cells to express more stem cell phenotype in a process called EMT transition (epithelial to mesenchymal transition) (5). In other words, differentiated cells that normally are designated to perform specific functions transform into an undefined phenotype

characterized by continuous growth and tissue invasion (Figure 3, panel B) (5).

SP-2577, a drug known as Seclidemstat, prevents LSD1's interaction in the NuRD complex, leading to the transcription of tumor suppressor genes (Figure 3, panel C) (5).

Seclidemstat is characterized as a potent noncompetitive reversible inhibitor of LSD1 with an  $IC_{50}$  of 13nM. (76). LSD1's dissociation in the regulator complex can prevent the stem cell-like phenotype of differentiated cells and lead to programmed cell death, or apoptosis (5).

It is reported that LSD1 can associate with other proteins involved in transcription co-activation and co-repression. In prostate cancer, LSD1 can activate gene transcription leading to the growth of tumors, mediated by the interaction with androgen receptors (6). All in all, preventing the demethylating and scaffolding forming capabilities of LSD1 can present itself as a potential target for anti-cancer and anti-tumor drug therapeutics in various forms of cancers.

### **1.3 Previous work on LSD1**

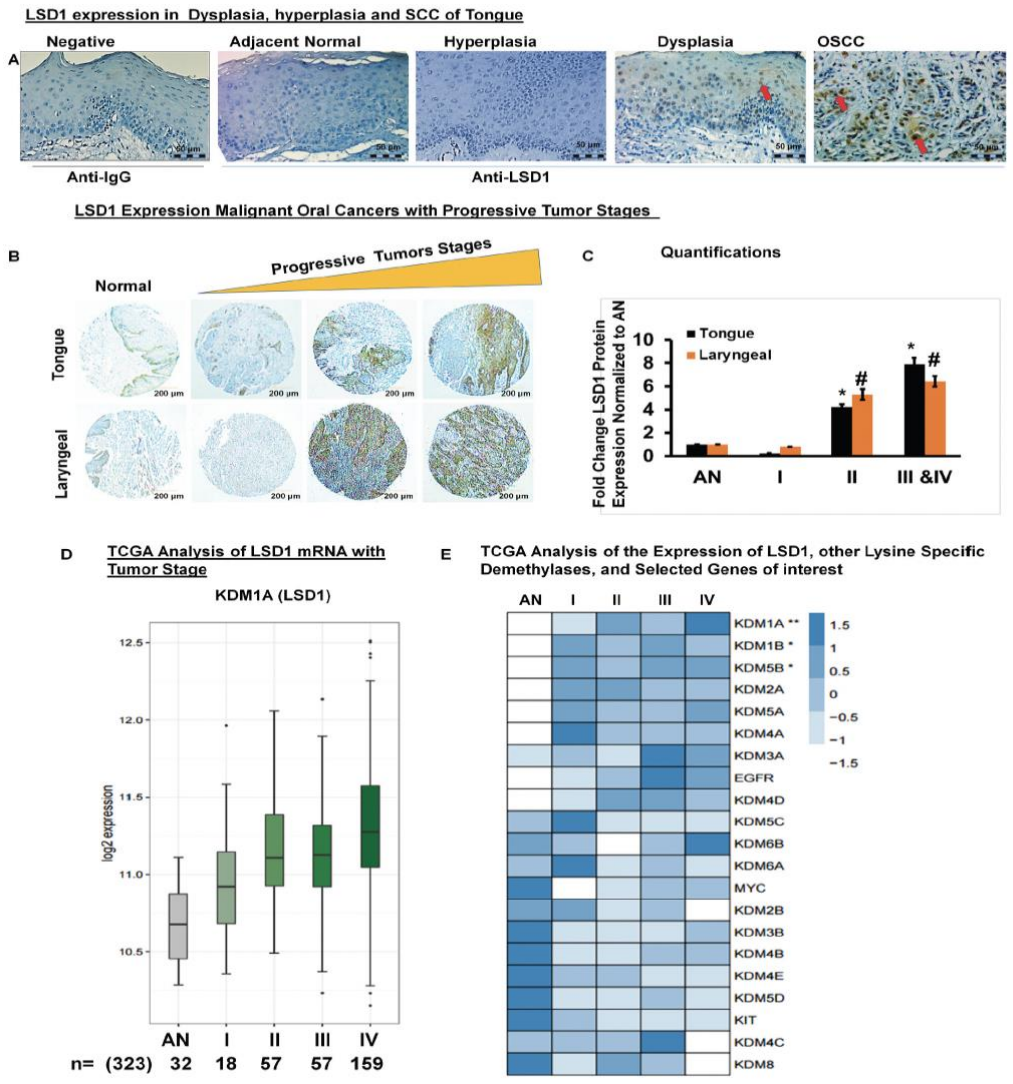
In our previous work, we have shown that LSD1 was able to promote growth and metastasis of human and mouse OSSC (7, 8). Furthermore, LSD1 expression was greater in clinical OSCC when compared to normal, hyperplastic and dysplastic human tissues which was shown by the immunostaining of LSD1 (Figure 4a). Subsequently, 80 OSSC

tissue samples were collected from various areas of the mouth including the larynx, tongue, and submandibular glands. These tissues exhibited varying grades and stages of OSSC and were subjected to tissue microarray analysis (Figure 4b). Staining of LSD1 was greater in tumors as the stages increased increasing stages. Staining of LSD1 was quantified and normalized to adjacent normal tissues (AN) (Figure 4c). The quantification of the staining show increasing amounts of LSD1 protein expression with increasing grade with the most intense staining noted in stage II, III and IV.

Bioinformatic analysis was performed on mRNA expression from The Cancer Genome Atlas (TCGA) consisting of more than 300 OSCC samples. Average LSD1 mRNA expression in tumor stages ranging from I to IV were compared to adjacent normal (Figure 4d). The data shown emphasizes that LSD1 expression increases with both tumor grade and stage. Figure 4E displays a heatmap of the expression levels of 21 genes of interest against LSD1 expression based on the TCGA analysis performed on the varying stages of tumors. *KDM1A* gene expression was upregulated in stage II and IV tumors.

The findings of these set of experiments highlight the important role of LSD1 in the regulation of OSSC. The detailed mechanism of LSD1's mechanism in HNSCC requires further investigation. Specifically determining how LSD1 induces epigenetic changes to transform normal oral epithelial to a state of malignancy warrant investigation.

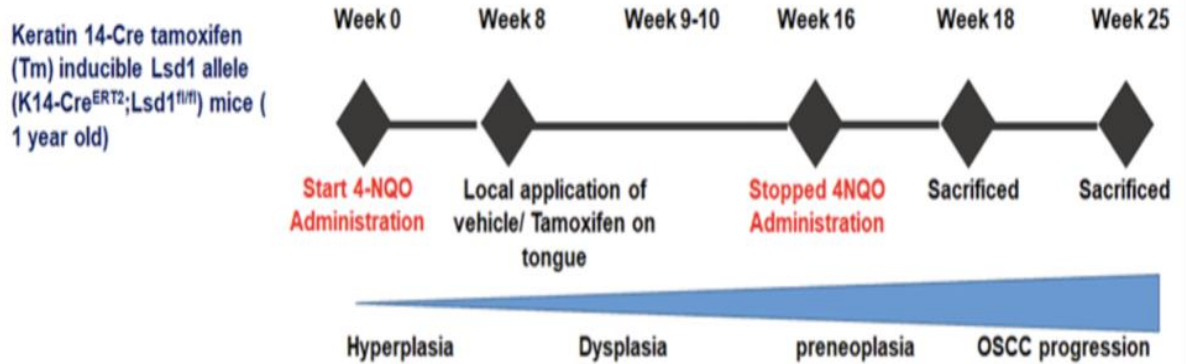
Furthermore, LSD1's role in promoting cancer stem cells, immune cells, signaling pathways and dynamic feedforward networks are unknown and should be explored.



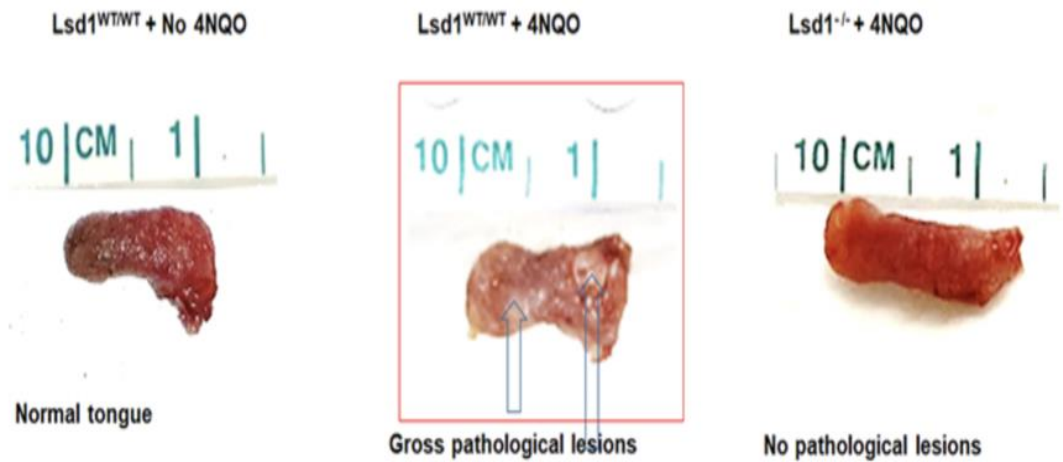
**Figure 4.** Aberrant expression of LSD1 in oral squamous cell carcinoma. Figure adopted from (7).

Previous work from our lab has also shown that genetic deletion of LSD1 attenuates OSSC.

**A** Experimental design



**B**



**Figure 5** Genetic deletion of LSD1 in Krt14-expressing cells results in reduced OSCC following 4NQO exposure. Figure adopted from (78).

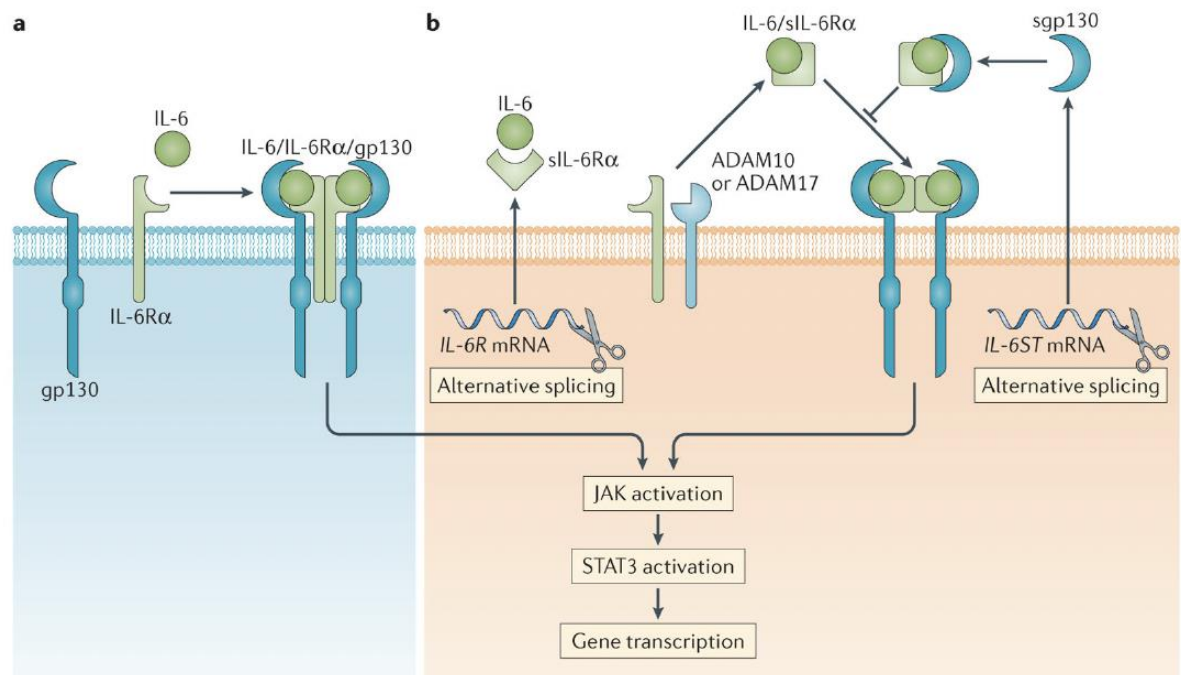
Figure 5a depicts the experimental design evaluating the role of LSD1 in the 4NQO mouse model. LSD1-floxed mice from Stuart Orkin laboratory (Mass General Hospital, Boston) were crossed with Cre recombinase expressing mice. In other words, a Keratin 14-Cre promoter specific tamoxifen inducible LSD1 knockout mice were created. This

allowed the deletion of the LSD1 gene in cells that expand in oral squamous cell carcinoma. In the mouse models 4NQO, a tobacco carcinogen was administered at week 0 to mimic pathologic changes like those seen in human OSSC (27-31). At week 8, local application of tamoxifen or vehicle was applied on the tongues of these mice. Mice were given 4NQO water at week 0 to 16 and were sacrificed at week 18 or 24. There were three groups of mice: wildtype that did not receive 4NQO water, wildtype that received the 4NQO water, and the LSD1 knockout group that received the 4NQO water. Mice that were from the LSD1 wildtype group with 4NQO showed gross pathological lesions with signs of pathologic changes (Figure 9b). In contrast, the LSD1 knockout mice showed a total absence or less severe gross pathological development of OSSC in comparison. The findings of this suggest that genetically deleting LSD1 in mice had some benefit in that no pathology was noted on these tongues. These tongue samples were used in further studies.

#### **1.4 IL-6/JAK/STAT3 pathway**

The IL-6/JAK/STAT3 pathway is one mechanism which was explored to help unravel the mechanism of LSD1's action in OSCC. The IL-6/JAK/STAT3 pathway is aberrantly hyperactivated in many cancers (11). Hyperactivation of Janus Kinase (JAK) and signal transducer and activator of transcription (STAT) have been shown to promote

tumorigenesis (12). This hyperactivation is often associated with poor clinical prognosis (11). The IL-6/JAK/STAT3 pathway play a key role in tumor microenvironments, driving proliferation, survival, invasion, and metastasis (11). Furthermore, this pathway also suppresses the body's antitumor immune responses (11).

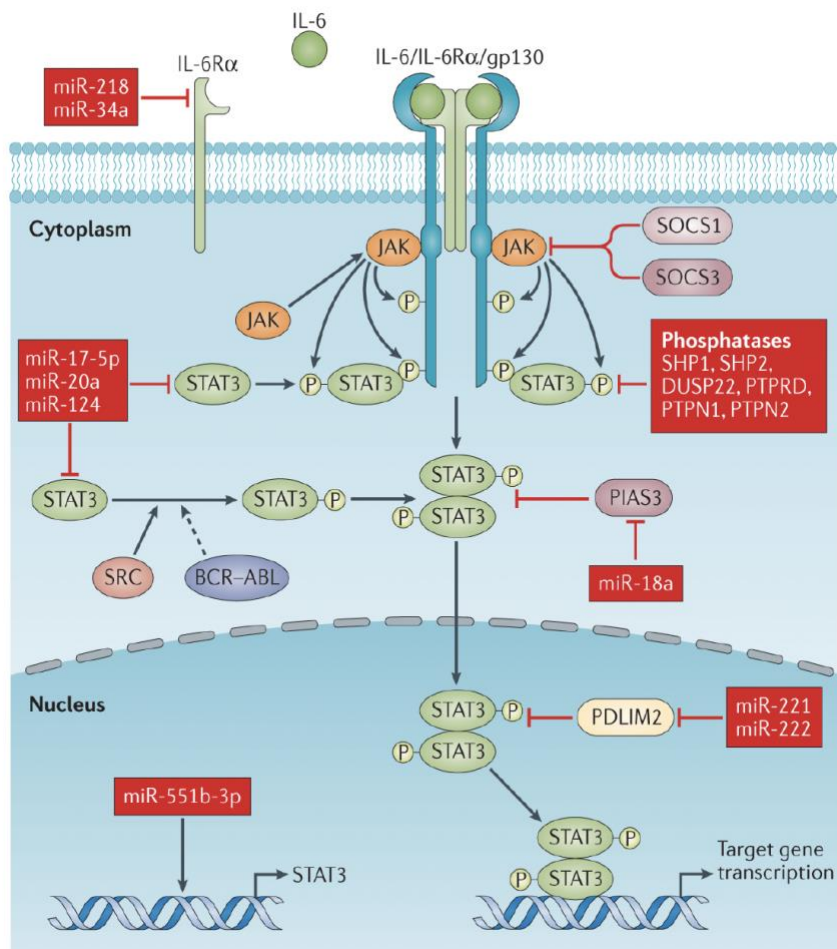


**Figure 5.** IL-6 Signaling pathways a) Classical pathway b) IL-6 *trans*-signaling pathway.

Figure adopted from (11).

Figure 5a outlines the classical IL-6 (interleukin-6) signaling pathway. IL-6 binds to its receptor IL-6R $\alpha$ , which is often referred to as IL-6R and forms a heterohexameric complex. This complex involves 2 molecules of IL-6, IL-6R and IL-6 receptor subunit- $\beta$ , also

referred to as gp130. Once these complexes form, it subsequently activates the JAK/STAT3 pathway inside the cell (Figure 6). Figure 5b outlines the IL-6 *trans*-signaling pathway involving soluble sIL-6R. sIL-6R is produced by alternative splicing of mRNA or by cleavage of membrane IL-6R by ADAM10 or ADAM17. Binding of IL-6 to sIL-6R results in dimerization of gp130 and the JAK/STAT3 pathway is activated (11).



**Figure 6.** Downstream signaling of the IL-6 receptor. Figure adopted from (11).

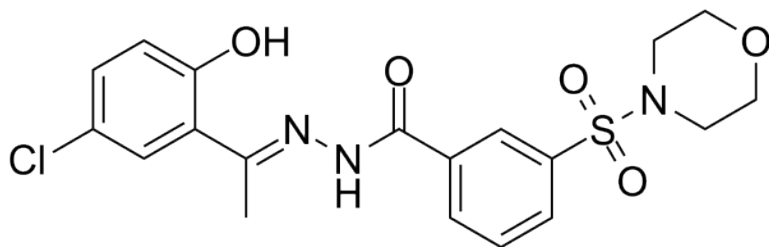
After the binding of IL-6 and the formation of the heterohexameric complex, the intracellular cascade is activated. Intracellularly, JAK proteins bind to gp130 at Box 1 and Box 2 sites. This binding initiates phosphorylation of gp120 at multiple tyrosine residue sites. Phosphorylation occurs at 4 tyrosine residues that STAT3 binds to, including an important site tyrosine 705. After phosphorylation, STAT3 forms a dimer and travels into the nucleus where it transforms into an active state (12). The STAT3 dimer then travels to the nuclear chromatin and induces transcription of certain genes.

As seen in Figure 6, this pathway can be regulated in different ways. Oncogenic proteins such as SRC and BCR-ABL1 can also induce the phosphorylation of STAT3, and subsequently, its dimerization and activation. Other molecules and proteins can also affect this pathway for example suppressor of cytokine signaling (SOCS) 1 and SOCS3. These proteins inhibit the phosphorylation capabilities of JAK in the upstream part of the pathway. Multiple phosphatases work to remove the phosphate group on certain proteins preventing the activation of this pathway as seen in the red box in Figure 6. Multiple endogenous ligases such as PDLIM2 and PIAS3 work to degrade STAT3, preventing gene transcription. MicroRNAs (miRNAs) can have a pro-oncogenic role by inhibiting ligases PDLIM2 and PIAS3 leading to activation of STAT3 and transcription of select genes. Other miRNA such as miR-218 and miR-34a can prevent the downstream activation of the IL6-JAK-STAT3 pathway by negatively regulating the IL6R protein.

The aberrant activation of STAT3 can lead to tumor cell survival, proliferation, angiogenesis, and metastasis (13, 14). Drugs that target STAT3 by regulating its phosphorylation can affect specific pro-tumor drug transcription, affecting cell cycle, proliferation, growth, and apoptosis (15). STAT3 has been an area of interest for target drug therapeutics in antineoplastic therapies (16). Targeting STAT3 signaling has already shown benefits in restoring anti-tumor immunity as a monotherapy or in combination therapies (25).

### **1.5 LSD1 Inhibitor SP2509**

Small molecule inhibitors have been developed to bind specifically and reversibly to LSD1 (18, 19). These potent drugs have a N'-(1-phenylethylidene)-benzo hydrazide structural group with an IC<sub>50</sub> (half maximal inhibitor concentration) of 200-400nM (18). SP2509, a selective LSD1 antagonist, exerts significant antitumor activity in a variety of cancers (20). SP2509 is a non-competitive inhibitor also called HCl2509 and LSD1-C12 (21). Its use has shown potent effects on AML cells *in vitro* and in xenografts (22). SP2509 has an IC<sub>50</sub> of 13 nM (the same as Seclidemstat) and its chemical structure is shown in Figure 7 (23). Its chemical structure is almost identical to Seclidemstat (SP-2577), with a slight substitution of a nitrogen group for an oxygen group in the rightmost ring structure.



**Figure 7.** Chemical structure of SP2509, a selective antagonist of LSD1 adopted from (23).

## 2. Hypothesis and Aims

The hypothesis of our study is that LSD1 promotes oral cancer progression through specific oncogenic signaling pathways. Furthermore, genetic, or pharmacological inhibition of LSD1 could attenuate these pathways for future translational applications.

To test this hypothesis, our specific aims are firstly: to determine if genetic or pharmacological inhibition attenuates oral squamous cell growth *in vivo*. Our second aim is to determine the mechanism of LSD1 action and its role in specific oncogenic signaling pathways such as the IL6/JAK/STAT3 pathway.

## 3. Materials and Methods

### 3.1 4-NQO Induced Oral Cancer Mouse Model

The animal experiments were approved by the Institutional Animal Care and Use Committee, Boston University. Mice from Jackson Laboratory that were from C57BL/6J strains were fed 4-Nitroquinoline 1-oxide (4NQO) drinking water (100ug/mL in propylene glycol and water). Mice were given this water for 16 weeks and then were given untreated water for the remainder of the experiment. A well-established protocol was followed (77).

### **3.2 Cre-LoxP System**

Transgenic mice were created by utilizing the Cre/loxP system. K14-Cre is a human promoter which induces the expression of Cre recombinase. The K14-Cre transgenic mice used the Cre-loxP system to genetically delete LSD1. This was done by crossing conditioned floxed mice (loxP-LSD1-loxP) with K14 promoter-driven tamoxifen inducible Cre mice (Jackson Laboratory). The conditional LSD1 knockout mice (K14Cre-*LSD1*; LSD1<sup>-/-</sup>) and littermates (LSD1<sup>WT/WT</sup>) were treated with 4-Nitroquinoline 1-oxide (4NQO) for eight weeks. Local application of either tamoxifen or vehicle on the tongues of these mice was performed for epithelial specific LSD1 deletion.

### **3.3 Inhibition of LSD1**

4NQO treated mice were subjected to LSD1 inhibitor (SP2509) at week 14. The

administration of SP2509 (in 25uL of corn oil, 5% DMSO) was applied topically on the mice tongues after brief anesthesia with 3% isoflurane. The dose of SP2509 was optimized to 40mg/kg three times a week for the duration of the experiment.

### **3.4 Cell Culture**

HSC-3 and CAL27 cell lines are the two metastatic and non-metastatic human oral cancer cell lines that were used, respectively. These low passage cells were grown in 100mm<sup>3</sup> plates to a confluency of less than 90% (approximately 20,000 cells per well). Cells were grown in a humidified incubator (37°C and 5% CO<sub>2</sub>). Cells were grown in DMEM (Dulbecco modified eagle's medium) from ThermoFisher Scientific. The media was supplemented with 10% HI-FBS (heat inactivated fetal bovine serum, F4135, Sigma) and 1% PS (Penicillin/Streptomycin).

Experiments which involved the administration of exogenous IL-6 involved the administration of 1ul of IL-6 with a concentration of 10ug/mL. The IL-6 administered is a form of recombinant human IL-6 (Biotechne) into the wells and were placed in the incubator for 30 minutes. These plates were then treated with either 0, 1 or 10uM of SP2509. These plates were returned to the incubator for 2 hours. The plates were removed, placed on ice and 0.5mL of lysis buffer was injected into each of the wells. The

wells were subsequently scraped with a cell lifter scarper for 2 minutes after which the proteins were collected.

### **3.5 RNA isolation and purification**

HSC-3 and CAL27 cell lines were treated with SP2509 and were placed into the incubator for 24 hours (some received exogenous IL-6 in the same manner described above). The plates were removed from the incubator, placed on ice and 1 mL of TRIzol reagent was added to each well. Total RNA was extracted based on the protocol by Invitrogen. RNeasy Plus Mini Kit by Qiagen was used to purify RNA following the manufacturers protocol. RNA concentration was measured using the NanoDrop spectrophotometer. Next, RNA was reverse transcribed into cDNA using the Quantabio SuperMix following the specified protocols.

### **3.6 Real Time qPCR/primers**

RT-qPCR was performed using primers ordered from ThermoFisher. RT-qPCR was performed using SYBR Green and StepOne RT-qPCR machines according to the standard protocol. The gene expression was normalized to the housekeeping gene GAPDH. Details of the primer sequences are listed in Supplemental Figure 4.

### **3.7 Western Blot**

Bicinchoninic acid Protein Assay (BCA) was used to quantify protein levels within the samples and were used to load equal protein amounts. Western Blots were performed using BIORAD protocols and equipment. Antibodies for STAT-3, P-STAT3 and beta actin were ordered from Cell Signaling and the appropriate primary and secondary antibodies were used at a recommended dilution of 1:1000 and 1:2000 respectively. ChemiDOC™ MP Imaging System by BioRad was using for western blot developing, imaging and quantification.

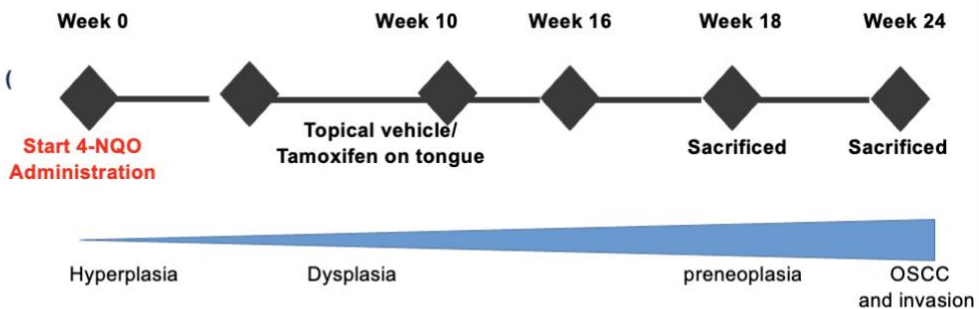
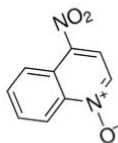
### **3.8 Statistical Analysis**

Student's t test, One-way ANOVA or Two-way ANOVA was performed using GraphPad Prism (San Diego) software version 7. P values less than 0.05 were considered statistically significant.

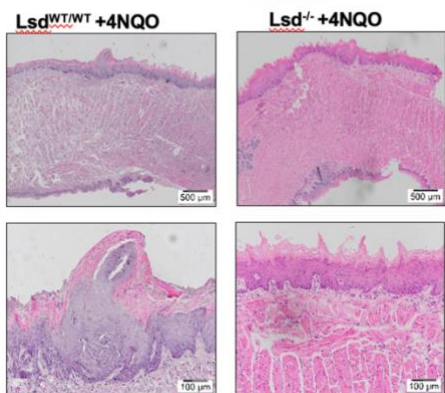
## **4. Results**

### **4.1 Oral epithelial specific genetic knockdown of LSD1 attenuates OSSC**

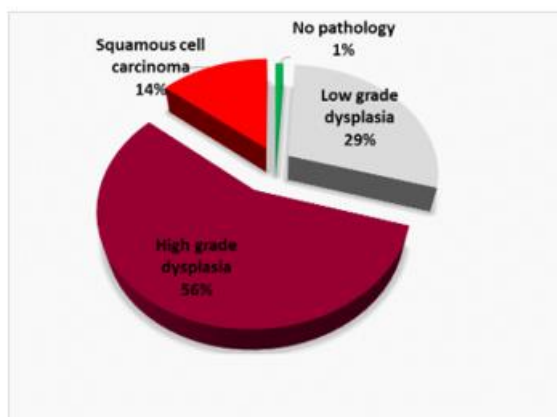
Keratin 14-Cre tamoxifen (Tm) inducible Lsd1 allele (K14-Cre<sup>ERT2</sup>;Lsd1<sup>fl/fl</sup>) mice (1 year old)



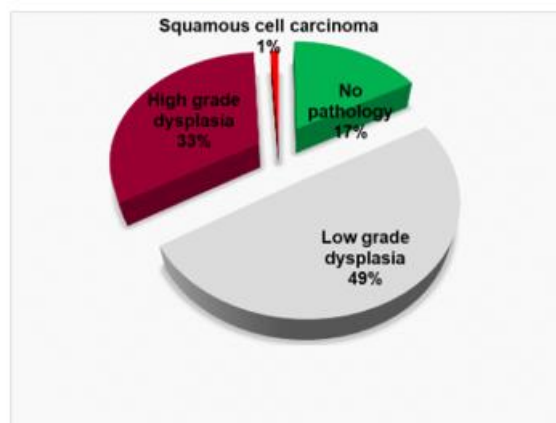
**Histopathological changes post-18 week 4NQO treatment**



**Lsd<sup>WT/WT</sup> +4NQO**



**Lsd<sup>-/-</sup> +4NQO**



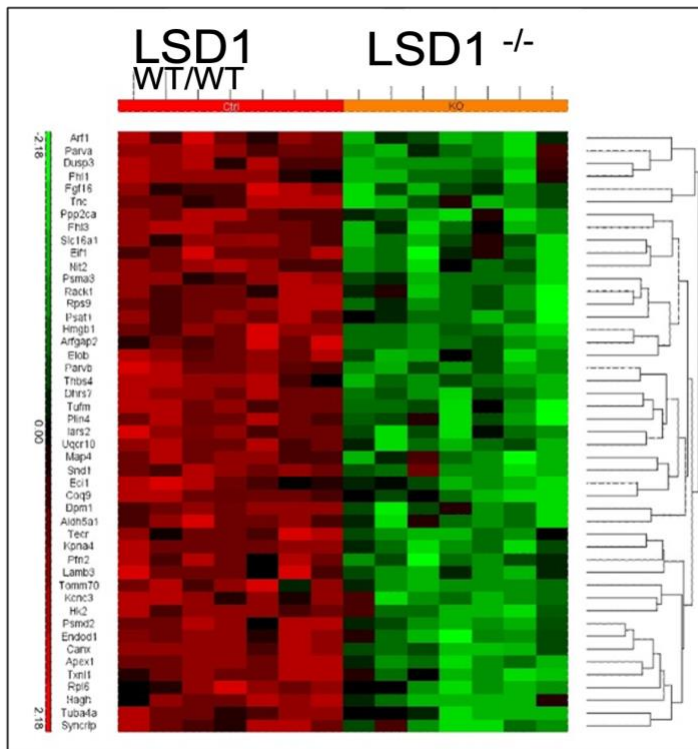
**Figure 9.** Genetic deletion of LSD1 in Krt14-expressing cells results in reduced OSCC following 4NQO exposure.

Figure 9 (top) depicts the experimental design evaluating the role of LSD1 in the 4NQO mouse model. LSD1-floxed mice from Stuart Orkin laboratory (Mass General Hospital, Boston) were crossed with Cre recombinase expressing mice. In other words, a Keratin 14-Cre promoter specific tamoxifen inducible LSD1 knockout mice were created. This allowed the deletion of the LSD1 gene in cells that expand in oral squamous cell carcinoma. In the mouse models 4NQO, a tobacco carcinogen was administered at week 0 to mimic pathologic changes like those seen in human OSSC (27-31). At week 8, local application of tamoxifen or vehicle was applied on the tongues of these mice. Mice were given 4NQO water at week 0 to 16 and were sacrificed at week 18 or 24. There were two groups of mice: wildtype that received 4NQO water and the LSD1 knockout group that received the 4NQO water. Mice that were from the LSD1 wildtype group with 4NQO showed gross pathological lesions with signs of pathologic changes (Figure 9). In contrast, the LSD1 knockout mice showed a total absence or less severe gross pathological development of OSSC in comparison. The pathology of the tongue were quantified and the wildtype mice had 56% high grade dysplasia, 29% low grade dysplasia, and 14% squamous cell carcinoma and 1% area of no pathology. In contrast,

the LSD1 knockdown group had 49% low grade dysplasia, 33% high grade dysplasia, 1% squamous cell carcinoma and 17% area with no pathology. The findings of this suggest that genetically deleting LSD1 in mice had some benefit in that no pathology was noted on these tongues. These tongue samples were used in further studies.

#### **4.2 Global Proteomics Signature shows downregulation of prooncogenic signaling proteins in 4NQO induced LSD1<sup>-/-</sup> vs LSD1<sup>WT/WT</sup> tongue tumors**

The first goal of the study was to evaluate the changes in the tumor microenvironment due to the inhibition of LSD1 oncogenic promoter. Mice tongue lysates from the experiment outlined in Figure 9 were sent for global unbiased proteomics analysis (after 4NQO treatment). The specific protein signatures and the heat map are shown in Figure 10.



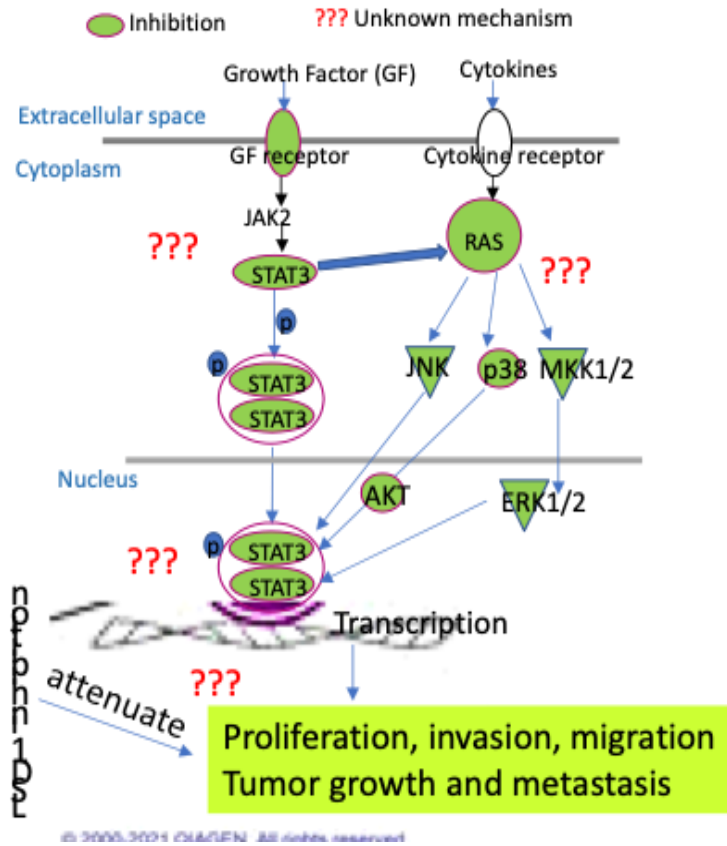
**Figure 10.** Global proteomics signature downregulated in 4NQO induced LSD1<sup>-/-</sup> vs LSD1<sup>WT/WT</sup> tongue tumors

In Figure 10, all the proteins in the LSD1 wild type tongue (left panel) were compared to the tongue of the LSD1 knockout mice (right panel). There were differences in the protein panel of mice with genetic deletion of LSD1 compared to the wildtype. Specifically, the data from the tongues with LSD1 specific deletion showed a downregulation of prooncogenic signaling proteins.

### 4.3 IPA Analysis shows that LSD1 inhibition attenuates the JAK-STAT3 pathway

The second goal of this study was to identify a specific pathway network in which LSD1 acts on. Ingenuity pathway analysis (IPA; Qiagen Inc) was performed to predict the potential pathways involved based on protein signature generated from the LSD1 knockout mice and control mice tongue protein lysates. IPA analysis showed that LSD1 inhibition attenuates the STAT3 network and specific co-interacting pathway network proteins (green color; Figure 11).

#### IPA analysis: LSD1 inhibition attenuate pathways

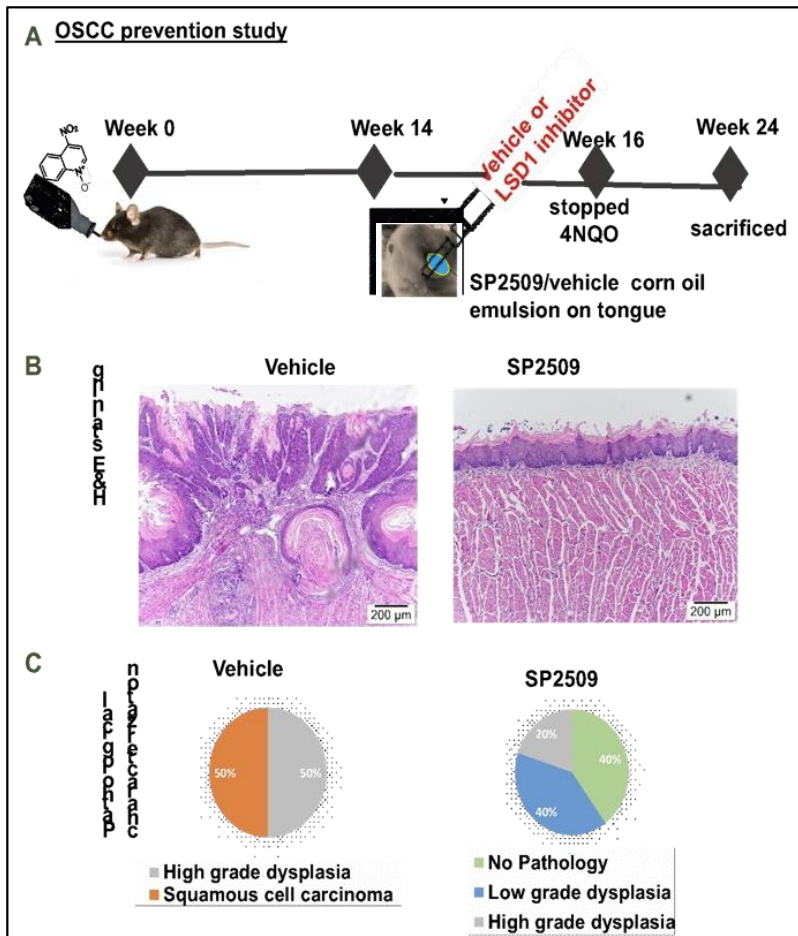


**Figure 11.** Ingenuity pathway analysis (IPA) of 49 protein signatures shows inhibition of JAK-STAT3 and co-interacting network proteins

Based on IPA analysis, we see that LSD1 inhibition appears to be attenuate the JAK/STAT3 pathway. From our previous discussion, the IL6-JAK-STAT3 pathway is activated in many types of cancers. If LSD1's mechanism of action involves the activation of IL6-JAK-STAT3 pathway, then it's inhibition may be a mechanism of inhibiting the IL6-JAK-STAT3 pathway in the cancer state. The details of this mechanism and attenuation warrant further investigation.

#### **4.4 Pharmacological inhibition of LSD1 with a small molecule inhibitor SP2509 attenuates oral cancer growth**

The previous mouse model experiment evaluated the genetic deletion of LSD1. The subsequent mouse experiment involved the pharmacologic inhibition of LSD1 by means of the administration of the small molecule inhibitor, SP2509. The effects on oral cancer were explored as seen in Figure 12.



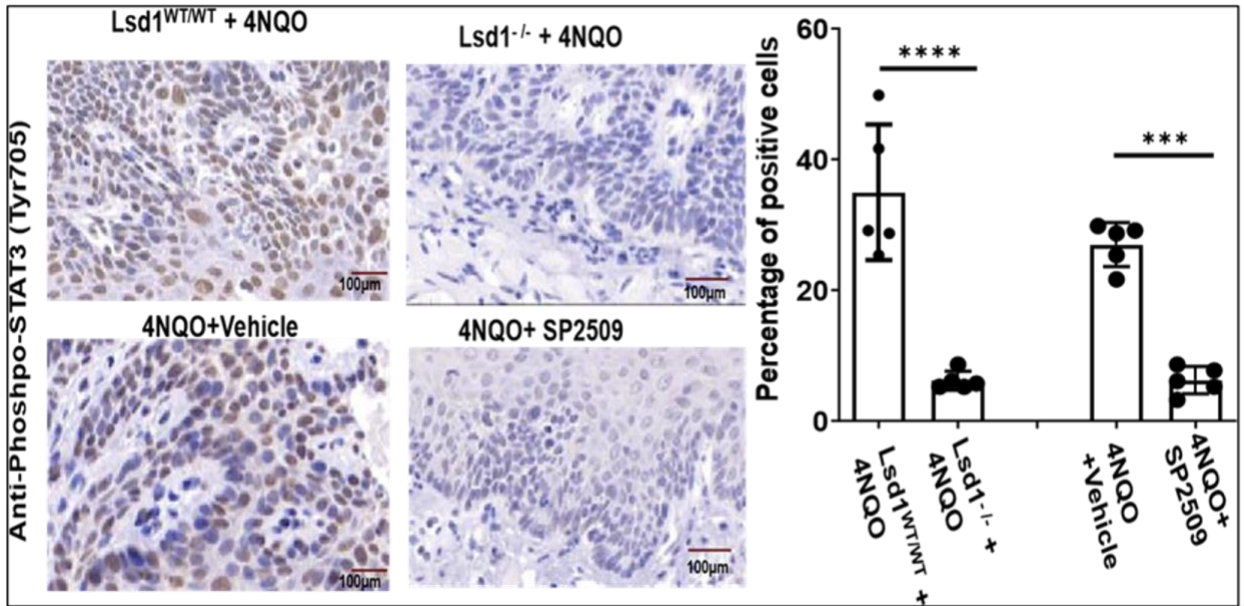
**Figure 12.** OSCC prevention study

In this OSCC pre-cancer mouse model, 4NQO was administered from week 0 to week 14. At week 14, the mice were fed either the LSD1 inhibitor SP2509 or vehicle. At week 16, the 4NQO was stopped and the mice were sacrificed at week 24. In Figure 12b, the hemoxylin and eosin staining on these tongue samples of the control mice displayed areas of dysplasia. The mice that received SP2509 (LSD1 inhibitor) showed unaffected

pathology. In Figure 12c, the pathology characterization found that the control group displayed about 50% high grade dysplasia and 50% squamous cell carcinoma. In contrast, the SP2509 group showed 40% of areas with no pathology, 40% with low grade dysplasia and 20% with high grade dysplasia. The main findings of this experiment suggests that the application of the small molecular inhibitor SP2509 attenuated the growth of oral cancer in a pre-cancer mouse model.

#### **4.5 Genetic or pharmacologic inhibition of LSD1 with a small molecular inhibitor (SP2509) attenuates STAT3 signaling pathways.**

The next goal was to evaluate if LSD1 inhibition attenuates STAT3 positive cells. We evaluated the tongue tumors after genetic deletion of LSD1 in knockout mice and in pharmacological inhibition of LSD1 via immunostaining (Figure 12).



**Figure 13.** Genetic or pharmacological inhibition of LSD1 attenuates phospho-STAT3 activation in tongue tumor lesions in mice.

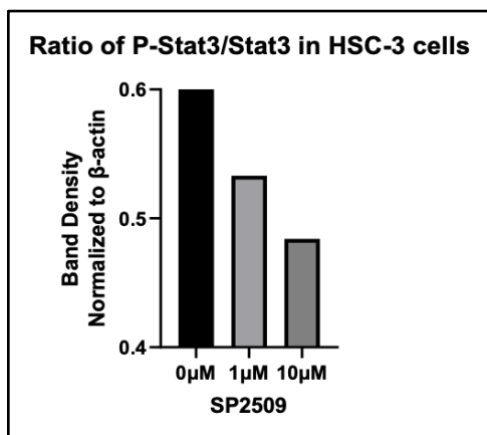
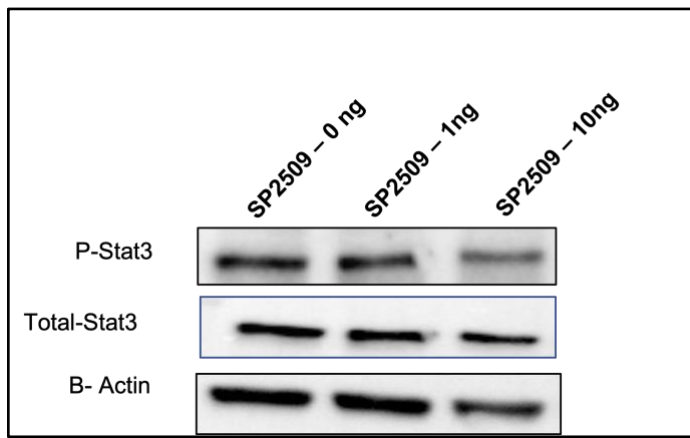
In the top panel of Figure 13, mice tongue samples from the genetic LSD1 deletion model were stained for Phospho-STAT3 at the tyrosine residue 705, an important docking site of STAT3 discussed earlier. Using anti-phospho-STAT3 antibodies, any areas of brown depict positive staining of P-STAT3. The LSD1 wildtype mice had significantly greater amounts of P-STAT3 staining when compared to the knockout group. This was quantified in the graph to the right. We see significantly lower percentage of positive P-

STAT3 staining cells in the wildtype relative to the group of mice that had tongue specific genetic deletion of LSD1.

In the lower panel of Figure 13, when LSD1 was inhibited by pharmacological means using an inhibitor SP2509, the control group again presented with a high degree of staining. In contrast, the staining from the samples that received the SP2509 inhibitor had less brown staining, implying less presence of P-STAT3. This was quantified in the right panel. The sample that received SP2509 had a significantly lower percentage of positive cells expressing P-STAT3 relative to the control.

The conclusion from this study is that genetic or pharmacological inhibition of LSD1 in pre-cancer attenuates STAT3 phosphorylation in mouse tongue tumors. We see a significant reduction of Phospho-STAT3, demonstrating that LSD1 inhibition blocks STAT3 immunopositive cells in the tumor microenvironment.

#### **4.6 Inhibition of LSD1 through attenuation of STAT3 phosphorylation in HSC-3 cell line *in vitro***



**Figure 14.** Inhibition of LSD1 through attenuation of STAT3 phosphorylation in HSC-3 cell line.

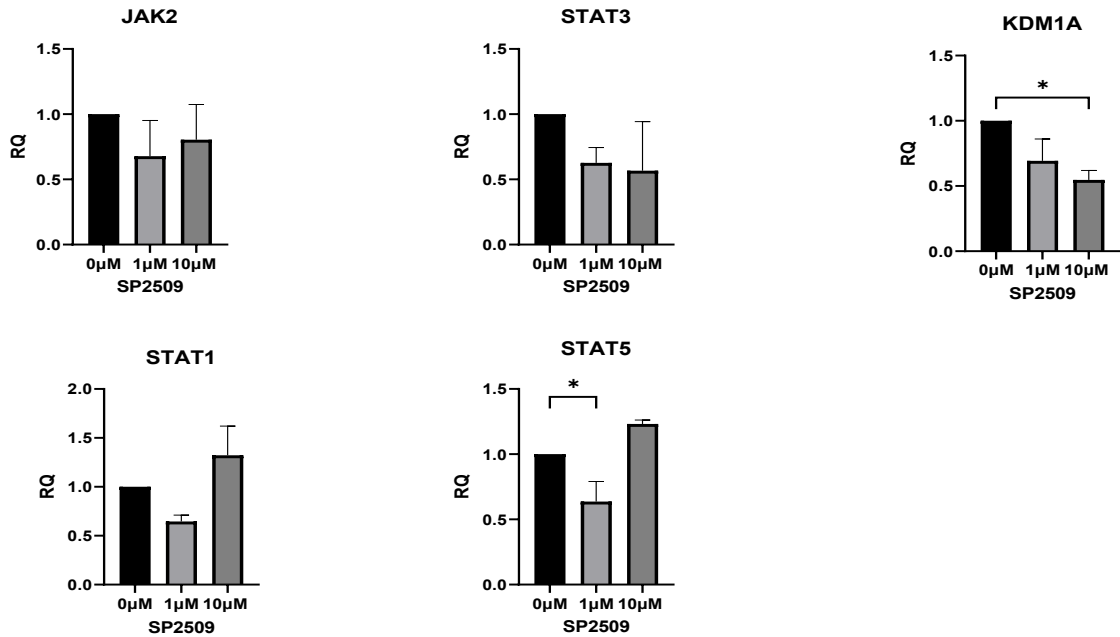
Human Squamous Cell Carcinoma (HSC-3) is a metastatic cell line that is highly aggressive (32). HSC-3 is a highly cited cell line used to study metastatic squamous cell carcinoma. It forms metastatic foci in draining lymph nodes when transplanted into nude

mice (33). HSC-3 cells have high levels of growth factor secretion, and their media has been shown to promote angiogenesis (32). HSC-3 cells were grown in 6 well plates to a confluence of 70% and varying doses of SP2509 were administered (0,1, 10ng) and incubated for 2 hours. When proteins were isolated from these experiments and subjected to western blot analysis, the amount of phosphorylated STAT3 decreased with increasing dose of SP2509. The western blot was quantified and Phospho-STAT3 and total STAT3 were normalized to Beta-Actin. The right panel of Figure 14 quantifies the results of the western blot and highlights that increasing dose of SP2509 decreased the ratio of P-STAT3 to total STAT3 in the HSC-3 cell line. In other words, SP2509 appeared to decrease the phosphorylation of STAT3 in a dose-dependent manner. These findings support the hypothesis that LSD1 may be attenuating oral cancer through the IL6-JAK-STAT3 pathway, specifically at the phosphorylation of STAT3 step.

#### **4.8 RT-qPCR showing mRNA expression of various genes after administration of increasing doses of SP2509 with and without exogenous IL-6 stimulation**

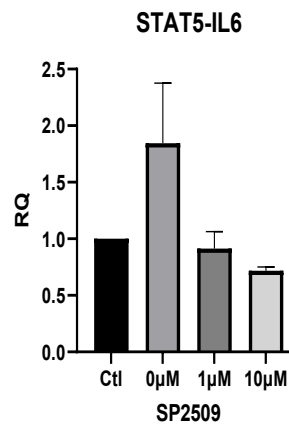
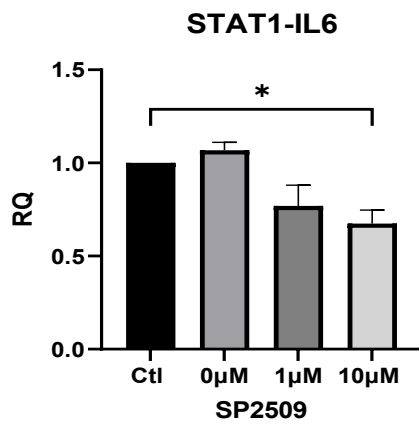
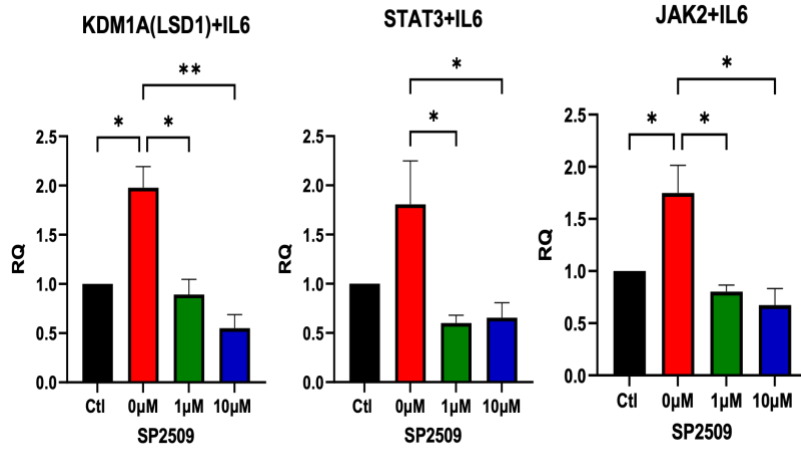
CAL27 was first established as an OSCC cell line in 1982 by Gioanni et al (34). This cell lines were established from a 56 male patient and tumor tissue was taken from poorly differentiated squamous cell carcinoma from the middle of the tongue (34). These cells

were transplanted into nude mice and their tumorigenicity was confirmed. CAL27 cell lines have been used in models to study OSSC for decades (35). CAL27 cells were grown in 6 well plates to a confluence of 70% and varying doses of SP2509 (0,1, 10ng) were administered followed by 24 hours of incubation. RNA was isolated from the experimental samples and subjected to RT-qPCR after RNA isolation purification and cDNA formation. Genes important in the IL6-JAK-STAT pathway were probed including JAK2 and STAT3. Additional genes investigated were KDM1A (LSD1), STAT1 and STAT5. One set of experiments involved no IL-6 stimulation (endogenous) and another set involved administration of IL-6 (exogenous) for 30 mins prior to application of the inhibitors. The exogenous IL-6 was administered to trigger the IL6-JAK-STAT3 pathway.



**Figure 16.** RT-qPCR shows mRNA expression of various genes after administration of increasing dose of SP2509.

The most notable findings from the experiment involving no exogenous IL6 displayed decreasing mRNA expression of KDM1A with increasing doses of SP2509. There was a statistically significant decrease in KDM1A expression with the administration of 10uM of SP2509 (\*p-value<0.05) (Figure 16).



**Figure 18.** RT-qPCR shows IL-6 stimulation of CAL27 oral cancer cells increases expression of KDM1A (LSD1), STAT3 and JAK2 followed by a decrease in expression with administration of SP2509.

The most profound findings from the set of experiments display that stimulation of CAL27 cell with exogenous IL6 increased the mRNA expression of KDM1A, STAT3 and JAK2 (Figure 18, red bar). This was then inhibited by increasing doses of SP2509 (green and blue bar). This suggests that the increasing dose of LSD1 inhibitor SP2509 decreased the level of mRNA transcripts of the KDM1A, STAT3 and JAK2 genes in a dose dependent manner (\*p-value<0.05). The mRNA levels of STAT1 and STAT5 were also noted to decrease with increasing dosage of SP2509.

## **5. Discussion**

### **5.1 STAT 3 in cancer**

The hyperactivation of the IL6-JAK-STAT3 pathway is seen in many cancers throughout the body and is often associated with poor prognosis (11). Many drugs that target IL6, IL6 receptors and JAKs have been approved by the FDA for inflammatory conditions and neoplasms (11). Their role and use are being investigated to treat malignancies and tumors. STAT3 selective inhibitors are novel and currently under study (11).

Elevated STAT3 activity has been estimated to occur in more than 70 percent of human cancers (36, 37). Hyperactivation of STAT3 have been seen in many malignancies including AML, multiple myeloma, tumors of the bladder, bone, breast, brain, cervix, colon, esophagus, kidney, liver, lung, ovary, pancreas, prostate, stomach, uterus and head and neck (38-51). Interestingly, phosphorylated/activated STAT3 have been correlated with poor prognosis (11). Active forms of STAT3 have shown tumor forming capacity of fibroblasts independent of anchorage, highlighting the oncogenic capabilities of STAT3 (52).

Altering the expression and function of STAT3 have shown importance in preclinical studies in the progression of many cancers (53-59). Even more surprising is that STAT3 can lead to resistance of chemotherapy, radiation, and monoclonal antibody therapeutics ex. Cetuximab (60, 61). Activation of STAT3 in a positive feedback loop can be a way that cells remain resistant to certain drug therapies (62). This suggests that targeting the activity of STAT3 in tumors may combat drug resistance in patients (11).

## **5.2 Inhibitors of STAT3**

Interestingly, STAT3 has been regarded as an “undruggable” target since it is a

transcription factor and does not have enzymatic activity (11). Developing inhibitors of STAT3 have been challenging, however some inhibitors are in clinical trials (63). These STAT3 inhibiting drugs are classified as STAT3 antisense oligonucleotides, STAT3 SH2 domain binders, and STAT3 decoy oligonucleotides (11).

In the context of head and neck cancer, a phase 0 trial was conducted in 2012 (64). STAT3 decoy oligonucleotides were injected into patient tumors and a decrease in expression levels of STAT3 target genes was noted (64). A cyclic version of the STAT3 decoy was created which reduced viability of cells, had an increased affinity to STAT3 and prevented the growth of xenografts (64). This study was novel because it was effective in inhibiting tumor STAT3 signaling. Another drug, C188-9 has been studied and is classified as a STAT3 SH2 domain binder (65). Its use is indicated in advanced stage and is undergoing phase I clinical investigation (65). It has shown to inhibit growth of radioresistant head and neck cancer xenografts (65).

### **5.3 Approved drugs for Treatment of Head and Neck Cancer**

The FDA approved two checkpoint inhibitors called Nivolumab (Opdivo®) and Pembrolizumab (Keytruda®) in 2016 (72). These drugs are targeted to squamous cell head and neck cancer patients who no longer respond to chemotherapy (72). These two drugs

are monoclonal antibodies and are classified as anti PD-1 as immune checkpoint inhibitors (73). Cetuximab (Erbix®) is an approved drug for the treatment of squamous cell head and neck cancer (74). It is also a monoclonal antibody and binds to human EGF receptors. Since it has a greater affinity to the receptor (by 5-10 times), it prevents the binding of endogenous growth factor EGF and prevents cancer cell growth and multiplication. It can also function in antibody dependent cell-mediated cytotoxicity (ADCC) by binding to EGFR, attracting immune cells that promote the death of these cancer cells.

#### **5.4 SP2509 as a therapeutic drug in cancer**

LSD1 inhibitors including TCP, ORY-1001, GSK-2879552, IMG-7289, INCB059872, CC-90011, and ORY-2001 are being investigated in clinical trials. Many of these drugs are being investigated for treatment of small lung cancer cells (SCLC) and acute myeloid leukemia (AML) (66). In addition to those listed, SP2509 has also shown promise for cancer treatment (66). Sharma et al. found that SP2509 had high potency and inhibited the proliferation of multiple cancer cell lines (67). Another study found that AML cells responded to SP2509 by off-target drug effects (68). SP2509 was found to allosterically target the H3 pocket of LSD1 which suppressed tumor growth in prostate cancer, independent of its demethylase function (69).

Another study found that treatment with SP2509 with Panobinostat inhibited AML blast progenitor cells and improved survival of NSG mice with established human AML (70). The combination of SP2509 with Panobinostat also improved the survival rates of mice with human AML cells and there was no toxicity noted (71).

Currently, there are only a select number of approved drugs for the treatment of head and neck cancer. Interestingly, none of them directly target the STAT3 protein. It would be interesting to see how targeting the IL-6/JAK/STAT pathway and specifically the activation of STAT3 may be an important site for drug intervention for the treatment of OSCC. Furthermore, if LSD1 is involved in the activation of STAT3, exploring the inhibition of LSD1 by means of an inhibitor such as SP2509 may have therapeutic benefits in the drug development for the treatment of HNSCC.

## **6. Conclusion:**

We showed for the first time that blocking LSD1 (a feed-forward loop oncogenic protein) inhibits OSCC, promotes anti-tumor immunity and attenuates STAT3 signaling *in vitro* and *in vivo*. Next, we expect to determine the detailed mechanism of LSD1 for application in chemotherapy and immunotherapy which could be mediated through STAT3. We are evaluating whether LSD1 inhibition attenuates STAT3 activation, translocation, or

transcription. Of note, STAT3 is a promoter of various oncogenic proteins and signaling networks. Nuclear translocation and activation of transcriptional programs could activate the oncogenic network. Overall, our preliminary studies are steps toward evaluating the mechanism of LSD1 in oncogenic progression and pathways involved in translational medicine.

## 7. Appendix

### Supplemental Figures:

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos:	Cursor abs	340 raw
water	Default	4/11/2023	4:51 PM	0.15	0.003	-0.011	-0.27	1	50	230	0.003	0.006
1 Sp-0		4/11/2023	4:53 PM	55.58	1.389	0.854	1.63	0.6	40	230	2.31	1.057
2 Sp-1		4/11/2023	4:55 PM	236.92	5.923	3.174	1.87	1.19	40	230	4.967	1.977
3 Sp-10		4/11/2023	4:56 PM	63.15	1.579	0.756	2.09	0.59	40	230	2.659	0.014
4 ORY-0		4/11/2023	4:56 PM	29.99	0.75	0.368	2.04	0.38	40	230	1.959	-0.002
5 ORY-1		4/11/2023	4:57 PM	425.16	10.629	5.32	2	1.72	40	230	6.175	0.762
6 ORY-10		4/11/2023	4:58 PM	148.7	3.718	1.888	1.97	1.08	40	230	3.454	0.725
7 Sp-0-IL6		4/11/2023	4:59 PM	177.59	4.44	2.109	2.11	1.77	40	230	2.513	0.046
8 Sp-1-IL6		4/11/2023	4:59 PM	136.36	3.409	1.669	2.04	1.42	40	230	2.407	0.246
9 Sp-10-IL6		4/11/2023	5:00 PM	30.76	0.769	0.427	1.8	0.74	40	230	1.039	0.391
10 ORY-0-IL6		4/11/2023	5:01 PM	27.33	0.683	0.342	2	0.98	40	230	0.699	0.019
11 ORY-1-IL6		4/11/2023	5:01 PM	73.75	1.844	0.882	2.09	1.92	40	230	0.961	0.007
12 ORY-10-IL6		4/11/2023	5:02 PM	27.13	0.678	0.316	2.15	1.2	40	230	0.567	0.007
1a	Sp-0	4/11/2023	5:03 PM	32.6	0.815	0.477	1.71	0.61	40	230	1.334	0.453
water	Default	4/11/2023	5:05 PM	0.43	0.011	-0.001	-10.56	0.61	40	230	0.017	-0.031

**Supplemental Figure 1.** Nanodrop data from the spectrophotometer after RNA isolation from SP2509 treated CAL-27 cells.

Primer Name	Orientation	Sequence
KDM1A	Forward	5'- CACCGAGTTCACAGTTAT TTAG
	Reverse	5'- TAGTTGGTAGGGGTTTTA TCC
STAT1	Forward	5'- ACCCAATCCAGATGCTA TG
	Reverse	5'- GAGCCTGATTAAATCTCT GG
STAT5	Forward	5'- ATGTGAAACCACAGATC AAG
	Reverse	5'- TCTGTGGGTACATGTTAT

		AGG
GAPDH	Forward	5'- TCGGAGTCAACGGATTT G
	Reverse	3'- CAACAATATCCACTTTAC CAGAG
JAK2	Forward	5'- CGACGGCAAATGTTCTG
	Reverse	5'- TGGATGTTCCCTCCATTT C
STAT3	Forward	5'- GGTACATCATGGCTTTAT C
	Reverse	5'- TTTGCTGCTTTCCTGAA TC

**Supplemental Figure 4.** Primer sequences of select genes investigated in RT-qPCR.

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**CURRICULUM VITAE**

